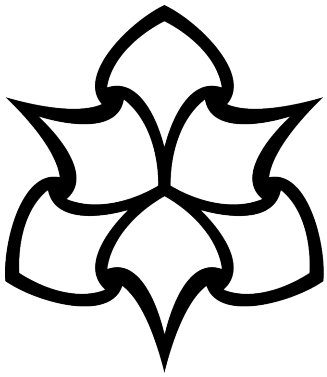

Metabolic adaptation of a *Chlamydomonas acidophila* strain isolated from acid mine drainage ponds with low eukaryotic diversity

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Abstract

The diversity and biological characteristics of eukaryotic communities within acid mine drainage (AMD) sites is less well studied than for prokaryotic communities. Furthermore, for many eukaryotic extremophiles the potential mechanisms of adaptation are unclear. This study describes an evaluation of eight highly acidic (pH 1.6 – 3.1) and one moderately acidic (pH 5.6) metal-rich acid mine drainage ponds at a disused copper mine. The severity of AMD pollution on eukaryote biodiversity was examined, and while the most species-rich site was less acidic, biodiversity did not only correlate with pH but also with the concentration of dissolved and particulate metals. Acid-tolerant microalgae were present in all ponds, including the species *Chlamydomonas acidophila*, abundance of which was high in one very metal-rich and highly acidic (pH 1.6) pond, which had a particularly high PO_4-P concentration. The *C. acidophila* strain named PM01 had a broad-range pH tolerance and tolerance to high concentrations of Cd, Cu and Zn, with bioaccumulation of these metals within the cell. Comparison of metal tolerance between the isolated strain and other *C. acidophila* strains previously isolated from different acidic environments found that the new strain exhibited much higher Cu tolerance, suggesting adaptation by *C. acidophila* PM01 to excess Cu. An analysis of the metabolic profile of the strains in response to increasing concentrations of Cu suggests that this tolerance by PM01 is in part due to metabolic adaptation and changes in protein content and secondary structure.

Keywords: *Chlamydomonas acidophila*, acid tolerance, metal tolerance, acid mine drainage, bioremediation, copper, zinc, cadmium

1. Introduction

Many freshwater bodies worldwide are highly acidic either due to natural causes or anthropogenic activities such as mining (Schultze, 2013; Smucker et al., 2014). Acid mine drainage (AMD) due principally to pyrite oxidation, is the cause of significant acidity in lakes and ponds situated in areas impacted by mining, and in rivers receiving mine water

discharge (Johnson, 2003; Nordstrom, 2000). Because decreasing pH causes increased solubility of metals, AMD results in high concentrations of dissolved Fe, S and various trace metals such as Cu, Cd and Zn in the contaminated waters. Concentrations of nutrients, especially inorganic phosphate (PO_4-P), are also frequently very low (Nixdorf et al., 1998). The combination of toxic metals and nutrient limitation limits biodiversity and can cause significant ecosystem damage (Deneke, 2000; Smucker et al., 2014). Evaluation of the biological impacts of AMD allows quantification of pollution damage, allows understanding of fundamental processes of adaptation and can identify AMD-tolerant species that have biotechnological applications, such as bioremediation (Ňancucheo and Johnson, 2011; Yun et al., 2014).

While prokaryotes in AMD environments has been extensively studied and reviewed (Johnson and Hallberg, 2003; Mendez-Garcia et al., 2015), there is still limited knowledge regarding the presence and roles of eukaryotes in these aquatic environments (Aguilera et al., 2006; Baker et al., 2004; Nixdorf et al., 1998). Photosynthetic microorganisms are found in many AMD ecosystems; however, the biodiversity of phytoplankton in such waters is severely limited and dominated by just a few acid-tolerant genera, such as *Chlamydomonas*, *Dunaliella, Euglena* and *Ochromonas* (Aguilera et al., 2006; Hargreaves et al., 1975; Ňancucheo and Johnson, 2012; Nixdorf et al., 1998; Pedrozo et al., 2001). Despite being able to tolerate the highly acidic and metal-rich conditions, productivity of these extremophile microalgae is often limited by low inorganic carbon and nutrient availability in acidic waters (Beamud et al., 2007; Spijkerman et al., 2007b). A fairly broad diversity of heterotrophic fungi and protists has also been observed in acidic waters (Baker et al., 2004; Das et al., 2009), while the diversity and abundance of zooplankton is typically very low as most species are unable to tolerate these environments (Deneke, 2000).

The high concentrations of dissolved metals in AMD can cause toxicity to microorganisms through a wide variety of mechanisms, some of which are shared between metals and across different organisms, such as competition with essential metals, direct interactions with proteins and other molecules within the cell, and induction of oxidative

77 stress (Sharma and Dietz, 2009). Metals such as Cu are particularly efficient at inducing the
78 formation of reactive oxygen species (ROS) in contrast to non-redox active metals such as
79 Zn and Cd (Valko et al., 2005). In most photosynthetic organisms, excess Cu has many
80 detrimental effects with the photosynthetic apparatus, including direct inhibition of
81 photosynthetic activity and degradation of chloroplast structures (Bernal et al., 2006; Küpper
82 et al., 2003). Furthermore, non-extremophile microalgae exposed to high Cu conditions
83 exhibit high concentrations of ROS and subsequent ROS-induced damage including lipid
84 membrane peroxidation (Jamers et al., 2013; Jiang et al., 2016; Sabatini et al., 2009).
85 The adaptive mechanisms by which eukaryotic microorganisms including
86 extremophile microalgae can survive in acid and metal rich conditions are still poorly
87 researched but potential insights into these mechanisms are increasing. For example,
88 proteomic approaches have indicated the importance of metal and acidity tolerance proteins,
89 such as molecular chaperones of the Heat Shock Protein family (Cid et al., 2010; Gerloff-
90 Elias et al., 2006). Likewise, genome sequencing and transcriptomics studies are beginning
91 to identify the array of genes that might explain extremophile functional characteristics, some
92 of which may have been obtained by horizontal gene transfer from bacteria. Genome
93 sequences of the acidophiles *Chlamydomonas eustigma* (Hirooka et al., 2017) and *Galdieria*
94 *sulphuraria* (Schönknecht et al., 2013) have recently been determined. Furthermore,
95 transcriptomic approaches are beginning to provide insight into the molecular mechanisms
96 of *Chlamydomonas acidophila* tolerance in response to Cd and Cu exposure (Olsson et al.,
97 2015; Puente-Sánchez et al., 2018), and *Dunaliella acidophila* in response to Cd (Olsson et
98 al., 2017; Puente-Sánchez et al., 2016), although further experimental analyses of these
99 transcriptomic datasets are needed.
100 AMD tolerant biota might have potential for bioremediation, with biological-based
101 processes potentially more cost effective and sustainable than chemical based methods
102 such as anoxic limestone drains and chemical addition (Geller and Schultze, 2013; Hedin et
103 al., 2010; Johnson and Hallberg, 2005). Bioremediation methods can include utilisation of
104 bacterial SO_4 reduction and neutralisation (Neculita et al., 2007) or aerobic wetlands that can

oxidise and precipitate dissolved metals (Dean et al., 2013). However, eukaryotic algae that can tolerate AMD conditions may be an alternative bioremediation agent (Abinandan et al., 2018; Das et al., 2009). Novel extremophile algal strains that show high acid and metal tolerance, and metal bioaccumulation traits are therefore needed for such applications. In addition, extremophile algae may have other biotechnological applications, such as a source of novel high-value chemicals including nutritional vitamins and anti-oxidants, food additives, and biofuels (Varshney et al., 2015).

The aim of this study was to identify eukaryotes, especially extremophile microalgae, in a series of standing waters affected by AMD with the intention to characterise a strain of microalgae for evidence of AMD adaptation. Following a screen of eukaryotic biota within nine Cu-rich AMD ponds, an extremophile chlorophyte microalgal strain identified as *C. acidophila* was examined in detail due to its abundance and ubiquity across the site and its high tolerance to acidity and dissolved metal concentrations, especially to Cu.

2. Materials and Methods

2.1. Study site

The site for this study is Parys Mountain, a disused Cu mine, in Anglesey North Wales, UK. The site has been mined for Cu from the Bronze Age, until mining activities ceased in the early 1900s (Dean et al., 2013). The area consists of large amounts of exposed spoil, with large pits and depressions that have filled with rainwater, and now retain large amounts of metal-rich and acidic water (Fig. 1). In addition, precipitation ponds and lagoons were constructed at the base of Parys Mountain, which were built in order to extract metals from the water as part of the mining process, and also contain large volumes of AMD polluted water (Younger and Potter, 2012). All the ponds are situated at close proximity within a similar geology with the rocks naturally rich in Cu, Pb and Zn. It is the only known example of Kuroko type volcanogenic massive sulfides in the UK, though the geology has been

disturbed by many millennia of underground and surface mining activities (Younger and Potter, 2012).

Of the various mining ponds and lagoons at the Parys Mountain site, nine ponds were examined (Fig. 1). Ponds 1 – 4 are located at an elevated position on the spoil outcrop, with one of these (Pond 4) on the side of a steep incline of the now drained large opencast (Fig. 1). The remaining five larger ponds (Ponds 5 – 9) are the precipitation ponds and lagoons at the base of the Parys Mountain outcrop, each adjacent to agricultural land. Ponds 1 – 4 and 9 are shallow and less than 1 m depth and subject to rapid variation in depth due to seasonal evaporation and rainfall. Ponds 5 – 8 are deeper and are typically 2 m in depth. All ponds showed little variation in depth across each pond. Ponds 1, 2 and 4 had no vegetation in or surrounding them, whereas the other ponds had surrounding vegetation and marginal wetland plants.

2.2. Field site sampling

Sampling at the nine AMD ponds was carried out in 2013 to 2015, including a spring (February and March), summer (June) and autumn (October) sampling regime in 2015. Water chemistry samples were taken in triplicate at each pond on each sampling occasion and were taken at approximately 15 cm depth 1 – 2 m from the edge of the pond. Water pH, conductivity, temperature and dissolved oxygen were measured using a YSI 556 probe (Xylem Analytics). For analysis of dissolved water chemistry metals 250 mL of pond water was filtered through a 0.45 µm cellulose acetate filter, as described previously (August et al., 2002; Boult et al., 1994), and a 50 mL volume was retained for the analysis of dissolved nutrients (PO_3-P, NO_3-N, NH_4-N). This dissolved fraction will also include some colloidal metals (Florence et al., 2016). A further 50 mL volume was acidified to 1% (v/v) nitric acid final concentration for the analysis of dissolved metals (Al, As, Cd, Cu, Fe, Mn, Pb, S, Zn). The filter paper was retained, and used for the determination of particulate metals.

For the analysis of algae 250 mL of unfiltered water was collected and aliquots were preserved with Lugol's iodine for enumeration of algae cells and further unfiltered water

sample was taken in a sterile container for the isolation and identification of algae. Algal samples were taken from all ponds, including samples taken from where algal biofilms were observed (Ponds 5 and 6). For chlorophyll-*a* measurement, 250 mL of pond water was filtered onto a GF/C filter paper.

Sediment samples were also taken to a depth of approximately 2 cm depth for determination of acid-extractable metals. For invertebrate sampling, sediment samples were taken from the littoral, approximately 1 m from the pond edge and 20 – 50 cm depth depending on the pond and sieved to remove organisms. This was followed by a 3 min sweep using a hand-held net with a mesh size of 1 mm, and a 3 min examination under large stones. Ethanol (70%) was added to preserve the invertebrate biota for identification to family level using standard keys (Greenhaigh and Ovenden, 2007; Quigley, 1977).

2.3 Nutrient, chlorophyll and metal analysis

Dissolved PO_3-P, NO_3-N, and NH_4-N were measured from the 0.45 µm filtered water samples using a Skalar Sans Plus autoanalyser. Chlorophyll-*a* concentrations were determined by absorbance spectroscopy following extraction in 96% (v/v) ethanol, as described previously (Dean et al., 2010). Dissolved metals were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Perkin-Elmer Optima 5300, exactly as described previously (Dean et al., 2013). Acid extractable sediment metals and suspended particulate metals were determined by acid digestion of 0.1 g of 250 µm sieved dried sediments and digestion of the pond water filter papers, respectively. Sediments and filter papers were digested in 5 mL ultrapure-grade nitric acid at 70°C for 4 h, diluted to 2% (v/v) nitric acid, and the metals measured by ICP-AES. Certified Reference Standard TM25.5 was used for all ICP-AES analyses. All samples were calibrated using a matrix-matched serial dilution of Specpure multi-element plasma standard solution 4 (Alfa Aesar) set by linear regression. Only results with a relative standard deviation < 20% were considered.

188 *2.4. Microalgae cultivation and analysis*

189 Microalgae was visually identified to genus level and enumerated from the Lugol's iodine
190 preserved water samples by light microscopy using a Sedgewick-Rafter cell counting slide
191 and a morphological taxonomy key (John et al., 2002). Isolation of individual algae strains
192 was carried out by incubating serial dilutions of water samples on modified acid medium
193 (MAM) agar plates at 22 °C with a 16-h light:8-h dark light regime and a photon flux of
194 approximately 150 µmol m^{-2} s^{-1}. MAM is a defined inorganic medium developed previously
195 (Olaveson and Stokes, 1989), and used here as described by the Canadian Phycological
196 Culture Centre, University of Waterloo, Canada containing 0.5 g L^{-1} $(NH_4)_2SO_4$, 0.01 g L^{-1}
197 $CaCl_2.2H_2O$, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.3 g L^{-1} KH_2PO_4, 0.03 g L^{-1} NaCl, 0.01 g L^{-1}
198 $Na_2EDTA.2H_2O$, 4.98 mg L^{-1} $FeSO_4.7H_2O$, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2.4H_2O$, 0.22
199 mg L^{-1} $ZnSO_4.7H_2O$, 0.39 mg L^{-1} $NaMoO_4.2H_2O$, 79 µg L^{-1} $CuSO_4.5H_2O$, 49.4 µg L^{-1}
200 $Co(NO_3)_2.6H_2O$, 1 µg L^{-1} vitamin B12, 1 µg L^{-1} biotin, and 0.2 mg L^{-1} thiamine-HCl, adjusted
201 to pH 3.0. Individual colonies were then extracted and grown in liquid MAM.

202 For identification of isolates using 18S rRNA gene amplification, DNA was extracted
203 using an UltraClean Tissue & Cells DNA isolation kit (Mo Bio) and 18S rRNA gene amplicon
204 sequences amplified using universal primers EukF and EukR (DeLong, 1992). DNA
205 amplicons were purified (Qiagen PCR Purification kit) before sequencing (to give ~1 kb
206 sequence reads) by GATC-Biotech, with subsequent sequence analysis performed by
207 BLAST, using the NCBI GenBank database (Table S1). The species identification of putative
208 *C. acidophila* strain was further confirmed by partial length 18S rDNA gene amplicon
209 sequencing, using PCR primers (18SFOR 5′-WAC CTG GTT GAT CCT GCC AGT-3′, and
210 18SREV 5′-GAT CCT TCY GCA GGT TCA CCT AC-3′) and PCR conditions as described
211 (Huss et al., 1999), and sequenced as described above but with sequence reads of ~1.7 kb
212 size. Phylogenetic analysis was then performed essentially as described previously
213 (Osundeko et al., 2013). 18S rRNA nucleotide sequences of selected unicellular Chlorophyta
214 microalgae of the *Chlamydomonas moewusii* clade were obtained from GenBank and
215 sequences were aligned using ClustalW. The phylogenetic tree was generated using the

maximum likelihood method using RAxML-GUI and the GTR-GAMMA model (Stamatakis, 2006). Confidence in the tree was assessed using the thorough bootstrap method by performing 10 runs of 100 replications.

An isolate of *C. acidophila* from Pond 1 (named PM01) was cultivated to quantify its tolerance to high metal concentrations and pH ranges alongside two strains of *C. acidophila* previously isolated from an acidic pond near Fratiskovy Lazne, Czech Republic (CCAP 11/136) (Fott and McCarthy, 1964), and an acidic mining Lake 111 in eastern Germany (CCAP 11/137) (Gerloff-Elias et al., 2005). All cultures were maintained in MAM pH 3.0. Cultures were serially inoculated in fresh media and were grown in batch culture conditions on an orbital shaker at 120 rpm at 22 °C with a 16-h light:8-h dark light regime and a photon flux of approximately 150 µmol m^{-2} s^{-1}. For metal exposure treatments, various concentrations of metals as chloride salts were added to liquid MAM pH 3.0 or solid MAM pH 3.0 agar plates. For pH range treatments, MAM was adjusted and buffered to the desired pH (from pH 1.0 to 7.0) as described previously (Gerloff-Elias et al., 2006). Essentially the medium was buffered through the presence of Fe in the medium for pH 1.0 to 3.0, with 10 mM citric acid for pH 4.0 to 5.0, and with 10 mM HEPES for pH 6.0 to 7.0. The pH could be maintained to within ~0.5 pH unit during the growth period. Starting cell densities were normalised by optical density measurement at 680 nm (OD_{680nm}). Algae growth was determined by cell number, and in some instances by OD_{680nm}, total chlorophyll or growth rate measurement, exactly as described previously (Osundeko et al., 2013). Algae samples were prepared for metal content measurement by ICP-AES, as described previously (Webster et al., 2011). Cells were EDTA washed to remove externally bound metals, as validated elsewhere for Cd, Cu, Pb and Zn (Hassler et al., 2004). Cells were centrifuged for 10 min at 3000 g, followed by resuspension of the cell pellet in 10 mL of 1 mM EDTA for 5 min, then re-centrifuged and washed with a 15 mL volume of Milli-Q water. After further centrifugation, cell pellets were oven-dried at 60 °C for 24 h and then digested in 0.5 mL of ultrapure concentrated nitric acid at 70 °C for 3 h. Samples were diluted in Milli-Q water to 2% (v/v) concentration of acid and analysed by ICP-AES as described above. Cells of *C.*

acidophila were measured (width and length) using an eyepiece graticule, and cell volume was calculated according the prolate spheroid formula (Hillebrand et al., 1999). The cell volume measurements were used to calculate the internal (cellular) concentration of metal (determined from EDTA-washed cells) on a volume basis. This value was then divided by the external (MAM or pond water) concentration of metal in order to calculate concentration factor (K_{conc}) values.

2.5. Fourier transform-infrared (FT-IR) spectroscopy

Each algal strain was grown in liquid MAM (pH 3.0), with Cu concentrations of 0, 6.5, 13 and 130 mg L^{-1} for 14 days, at which point cultures had an OD_{750nm} of between 0.3 and 0.5. Cultures were normalised to an OD_{750nm} of 0.3 and 10 mL was centrifuged at 1800 *g* for 5 min. The supernatant was removed and the cells washed in 1.5 mL of 0.9% (v/v) NaCl, then centrifuged and washed again before final resuspension of the cell pellet in 1 mL of 0.9% (v/v) NaCl before 20 µL of this suspension was deposited onto a 96-well silicon microplate. The samples were then oven dried at 40°C for 1 h, and an additional 20 µL sample was added and the samples once again dried at 40°C for 1 h. The plate was placed in a HTS-XT high-throughput microplate extension and FT-IR spectra collected using an Equinox 55 FT-IR spectrometer (Bruker Corporation), equipped with a deuterated triglycerine sulphate detector. Spectra were collected over the wavenumber range 4000-600 cm^{-1}. Each sample was analysed as nine technical replicates. Spectra were pre-processed using extended multiplicative signal correction (Martens and Stark, 1991) prior to multivariate analysis. Band assignments were determined as described previously (Driver et al., 2015).

2.6. Modelling and statistical analysis

Metal speciation modelling was performed using Visual Minteq version 3.0 (Gustafsson, 2010). All data was statistically analyzed by one-way ANOVA using Tukey post-hoc test performed using Prism v.6.04 (GraphPad). Principal component analysis (PCA) of environmental data was performed using PRIMER v.6 (Primer-E) and plotted using XLSTAT

(Addinsoft), while PCA of FT-IR spectra was performed using MATLAB version R2016a. All environmental data (except for pH values) were natural log transformed for PCA and linear regression analysis.

3. Results

3.1. Chemical characteristics of extremely acidic metal-rich mining ponds

The nine ponds were situated in close proximity (Fig. 1) but differed in water chemistry (Fig. 2). In all ponds, concentrations of suspended particulate metals were present at much lower concentrations compared to dissolved metals (Fig. S1). None of the metal or nutrient concentrations in the ponds differed significantly across the spring, summer and autumn samples. Ponds 1, 2 and 4 were situated on top of the Parys Mountain site in depressions of the spoil waste. These ponds were characterised by very low pH (< 2.0) and high conductivity values (Fig. 2A). Pond 1 in particular was highly acidic (mean pH of 1.6) and had a very high conductivity (mean 10.03 mS), which is predominantly controlled by the high dissolved S concentration, as well as high concentrations of dissolved Fe, Al, Cd, Cu and Zn (Fig. 2B, C). In addition, Pond 1 also had a very high PO_4-P concentration (Fig. 2D) and the highest dissolved As concentration (Fig. S1). Ponds 2 and 4 also had high dissolved metal and S concentrations but differed from Pond 1 due to lower conductivity (~3 mS) and much lower PO_4-P. Pond 3 was also situated on the top of the mine site, however, this pond stood out as the least polluted, with a weakly acidic pH (mean pH 5.3), and low conductivity and dissolved metals and S (Fig. 2A; Fig. S1). The precipitation ponds and lagoons (Ponds 5 – 9) were also highly acidic but had lower conductivity due to much lower dissolved metal concentrations, with the exception of dissolved Mn (Fig. S1). In most ponds, ammonium (NH_4-N) was the dominant form of N rather than nitrate (NO_3-N), as is expected in acidic waters where nitrification rates are low (Baffico et al., 2004). Pond 6 had unusually high NO_3-N (Fig. 2D) possibly because the pond is directly adjacent to fertilised farmland. Chlorophyll-*a* was detectable in all ponds, indicating the presence of photosynthetic

300 microorganisms, but Pond 1 also differed from the other ponds with respect to having
301 significantly higher concentration of chlorophyll-*a*, which increased substantially during the
302 year as the water temperature and insolation increased (Fig. 2E).
303 The differences in water chemistry between the ponds situated within the mine spoil
304 waste (Ponds 1, 2 and 4), the lagoons and precipitation ponds (Ponds 5 – 9), and the less
305 polluted Pond 3, are reflected in the PCA plot (Fig. 3). In particular, Pond 1 and Pond 3 were
306 distinguished from each other and from the other ponds through difference in pH.
307 Furthermore, the high sediment Al and Mn concentrations explained the differentiation of
308 Pond 3, likely a consequence of the high pH of this water resulting in the precipitation of
309 dissolved Al. Pond 1 was situated within the PC space particularly on the basis of high
310 chlorophyll-*a*, PO_4-P and dissolved As concentration. It is therefore apparent that many of
311 these ponds are highly toxic environments, particularly Pond 1 with its very high acidity and
312 high concentration of toxic dissolved trace metals including As, Al, Cd, and Cu.
313
314 *3.2. Taxa diversity*
315 Overall taxa diversity, as determined by number of different taxonomic families, was lowest
316 in the highly acidic, high conductivity Pond 1 (with two taxa) and also low in the Ponds 2 and
317 4, which are also situated among open mine spoil. The highest number of taxa were found in
318 the low conductivity, mildly acidic (pH 5.3) Pond 3, with 15 taxa, while the
319 lagoons/precipitation ponds had intermediate taxa diversity (7-10 families). Linear regression
320 analyses of the relationships between taxa diversity and water chemistry (Fig. S2) showed a
321 significant positive correlation between increasing pH values and increasing taxa number (R^2
322 = 0.59; p = 0.03) but also a very strong negative correlation between decreasing conductivity
323 and increasing taxa number (R^2 = 0.85; p = 0.001), indicative of the dissolved metal
324 concentration. In particular, there was significant correlation between dissolved As, Cd, Cu,
325 Fe and S and taxa number, as well as between particulate Fe and S with taxa number, but
326 no significant correlation on the basis of sediment metal concentration (Fig. S2).

Invertebrates were observed in all ponds, though only Chironomidae was identified in all, although there were only one or two individuals recorded from Pond 2 and 4 (Fig. 5A). In Ponds 1 and 4, this was the only invertebrate taxa present, indicating that the conditions in these ponds are not suited for a diverse invertebrate community, but are able to support a few species that have adapted to the extreme AMD conditions. Other invertebrate taxa that were abundant are Corixidae, present in five of the ponds, and Sialidae, present in four of the ponds, but both absent in the three most acidic ponds (Fig. 4). The observed biota in Pond 3 included 14 invertebrate families, including many that are pollution-sensitive. Ponds 5 – 9 contained between 5 and 7 invertebrate taxa. All ponds were devoid of macrophytes within the open water.

A number of distinct eukaryotic microorganisms were isolated from the open water including microalgal, fungal and protozoan species (Table S1). The highest eukaryotic diversity was in Ponds 2, 5 and 6 (Fig. 4). In Ponds 5 and 6 and their adjacent channels much of the microorganisms were observed to be associated in biofilms. These included a strain which showed 99% identity based on the 18S rRNA sequence to *Euglena mutabilis* (Table S1), a well-known acid-tolerant species that has been previously observed in AMD environments, including coal mine waste sites (Brake et al., 2001), and including at the adit draining from the Parys Mountain mine (Ňancucheo and Johnson, 2012). In addition, a non-motile chlorophyte (likely to be *Koliella corcontica*; 96% identity) that has been previously observed in mildly acidic lakes (Vrba et al., 2003), and a diatom (likely to be *Eunotia naegelii*; 98% identity) was found associated with biofilm adjacent to Pond 6. However, diatoms were not found in open water in any of the Parys Mountain ponds. Ponds 1, 3 and 7 had the lowest number of eukaryotic microorganisms, with just one chlorophyte algae taxa identified in each pond (Fig. 4). There was substantial heterogeneity between the ponds, with all microbial taxa present in just one pond sample with the exception of the *Euglena* sp. present in two ponds, and a *Chlamydomonas* sp. identified in all ponds (100% abundance). Sequencing of the ~1 kb 18S rRNA gene amplicon from the *Chlamydomonas* sp. showed highest sequence identity (99%) to a strain annotated as *C. acidophila* (CCAP 11/134)

(Table S1). Quantification of cell density of this strain found highest density in Pond 1 (Fig. 5A), which correlated significantly with the highest pond PO_4-P concentration ($R^2 = 0.60$; $p = 0.02$), but cell density was also lowest in the most alkaline pond water where there was the lowest dissolved metal concentrations, likely explaining the positive correlation between cell density and conductivity or dissolved metal concentrations, such as Cu (Fig. 5B).

3.3. Phylogeny of a *C. acidophila* strain

C. acidophila was found across the Parys Mountain site in all nine ponds and was highly abundant in the most acidic and metal rich Pond 1. Therefore this strain was studied further in more detail. The isolate of *C. acidophila* from Pond 1 (named PM01) was examined by longer (1.7 kb) 18S rRNA gene read sequence and phylogenetic analysis (Fig. 6). PM01 18S rRNA sequence was identical apart from one nucleotide within this 1.7 kb region to sequences from three strains annotated as *C. acidophila* (Gerloff-Elias et al., 2005): CCAP 11/134 (an isolate from Argentina), CCAP 11/136 (an isolate from Czech Republic) and CCAP 11/137 (an isolate from Germany). PM01 was also identical within this 18S region to an unidentified strain (Rt1n1) originally isolated from the acidic Rio Tinto river in Spain (Amaral Zettler et al., 2002). The CCAP 11/136 strain (Fott and McCarthy, 1964), previously regarded as an authentic strain of *C. acidophila* (Gerloff-Elias et al., 2005), is also deposited in a different culture collection as strain UTCC 354 (Pollio et al., 2005), but this has distinct 18S rDNA sequence (Fig. 6), indicating that CCAP 11/136 and UTCC 354 are not in fact identical. It was previously argued that the UTCC 354 strain assigned as *C. acidophila* may be more appropriately assigned as *Chlamydomonas pitschmannii*, another highly acidotolerant species (Pollio et al., 2005). Other strains recorded as *C. acidophila* including UTCC 121 (an isolate from Canada) (Twiss, 1990) and OU 030/a (an isolate from Japan) (Nishikawa and Tominaga, 2001), were also distinct from PM01 and CCAP 11/136 but grouped more closely to the UTCC 354 strain and *C. pitschmannii* (Fig. 6). Although strain OU 030/a has also been previously regarded as an authentic strain of *C. acidophila* (Nishikawa and Tominaga, 2001; Pollio et al., 2005), here we refer to the CCAP 11/136 and

11/137 strains as *C. acidophila* species, in line with previous analysis (Gerloff-Elias et al., 2005), and thus the PM01 strain is further referred to as a strain of this species.

3.4. AMD tolerance by *C. acidophila* PM01

To examine the relationship between pH and *C. acidophila* further, strain PM01 from Pond 1 was grown in MAM artificial pond water at a range of pH values from pH 1.0 to 7.0. PM01 exhibited a very broad pH tolerance range, with optimal growth at pH 3.0 – 5.0 (mean growth rate ranging between 0.158 – 0.175 d^{-1}; no significant difference between treatments; $p > 0.05$), and with the highest cell density after 25 d obtained in pH 3.0 conditions. Strong growth was still observed in pH 7.0 (mean growth rate 0.153 d^{-1}) and pH 2.0 (mean growth rate 0.157 d^{-1}) conditions, but with a significant reduction ($p < 0.05$) in growth after 25 d in pH 2.0 and pH 7.0 by 25% and 37%, respectively compared to pH 3.0 MAM. and could grow at pH 1.0 after an extended lag phase of 13 d (growth rate 0.018 d^{-1}), but with a significant reduction in growth by 73% after 25 days compared to pH 3.0 MAM. The pH characteristics of the ponds may explain in part the microalgae biodiversity and cell density profiles, and as described above, there was a significant negative correlation ($R^2 = 0.57$; $p = 0.02$) between pH and *C. acidophila* cell density (Fig. 5B).

Samples of PM01 taken directly from Pond 1 showed high concentration of absorbed and internalised metals, as determined by measurement of EDTA-washed cells, to remove externally cell wall-bound metals. There was a high concentration of Cu and Zn accumulated almost entirely within the cell (no significant difference between EDTA washed versus unwashed cells) (Table 1). Relative to mean Pond 1 dissolved Cu concentration of 58.6 mg L^{-1}, the internal cellular Cu concentration in pond 1 cells was 84.3 fg $cell^{-1}$ (equivalent to 185.5 mg L^{-1}). The mean concentration of dissolved Zn in Pond 1 was 37.9 mg L^{-1}, while the internal cellular Zn concentration was 115.0 fg $cell^{-1}$ (253.0 mg L^{-1}). This gives concentration factor (K_{conc}) values of 3.2 for Cu and 6.7 for Zn. Substantial bioconcentration was also observed for Pb and Cd. Relative to a mean Pb concentration in Pond 1 of 0.8 mg L^{-1}, the accumulation and bioconcentration of Pb by the strain was particularly high, with a cellular

411 concentration of 146.3 fg cell^{-1} (321.8 mg L^{-1}) giving a K_{conc} value of 421.8. Cd was also
412 accumulated almost entirely within the cell to a concentration of 2.8 fg cell^{-1} (6.2 mg L^{-1}), and
413 relative to pond water concentration of 0.3 mg L^{-1} gives a K_{conc} value of 24.5. In contrast,
414 only 56% of accumulated Fe was taken up into the cell (Fig. S3).

415 Metal tolerance and accumulation by *C. acidophila* PM01 was further examined in an
416 artificial growth medium to assess the tolerance range of three of the trace metals found
417 within the Parys Mountain ponds. PM01 was grown in increasing concentrations of Cd, Cu
418 and Zn in MAM at pH 3.0. For cells grown under controlled conditions, the maximum cell
419 density achieved and the total chlorophyll concentration per cell (as a measure of cell
420 physiological status) was higher than that observed for the cells analysed *in situ* (in Pond 1)
421 (Table 1). The PM01 strain displayed tolerance to high concentrations of these three metals.
422 To allow comparison with Pond 1 water concentrations, the free ionic Cd^{2+}, Cu^{2+} and Zn^{2+}
423 concentrations were calculated using the Visual Minteq speciation model. PM01 could
424 tolerate up to 2.6 mg L^{-1} Cd^{2+}, which was 16-times higher than present in Pond 1 water, with
425 no significant inhibition of growth rate, maximum cell density or chlorophyll content when
426 compared to no added Cd^{2+} (Table 1). However, at a concentration of 6.0 mg L^{-1} Cd^{2+} all
427 three parameters (growth rate, cell density, chlorophyll content) were significantly reduced (p
428 < 0.05). PM01 was highly tolerant to Cu, and none of the concentrations up to 78.3 mg L^{-1}
429 Cu^{2+} treatment significantly inhibited growth or chlorophyll-*a* concentration, the apparent
430 reduction in cell density was not significant. Substantial Zn tolerance was also observed,
431 with no significant inhibition to growth rate at 855.4 mg L^{-1} Zn^{2+}, which was nearly 30-times
432 higher than the Zn^{2+} concentration in Pond 1, but at 1760.8 mg L^{-1} Zn^{2+} there was a
433 significant ($p < 0.05$) reduction in maximum cell density, although growth rate and chlorophyll
434 concentration was not significantly inhibited. The strain was still growing in 3002.8 mg L^{-1}
435 Zn^{2+} despite cell density being inhibited by 94%, with also a significant ($p < 0.05$) reduction in
436 growth rate and chlorophyll concentration (Table 1).

437 Accumulation of Cd, Cu and Zn was also quantified in the metal-treated PM01 strain
438 after 25 d growth in MAM pH 3.0 (Table 1). There was a concentration-dependent increase

in cellular accumulation of Cu and Cd with no significant difference between the values with or without EDTA washing, indicating that almost all of the Cu and Cd was taken up within the cell. In contrast, there were significant ($p < 0.05$) differences in Zn concentration following EDTA washing, indicating that a smaller proportion of Zn was internalised (Table 1). As the Zn concentration in the medium increased, the relative concentration within the cell decreased, suggesting that Zn transport into the cell saturated at higher concentrations. Overall, the characteristics of metal accumulation in artificial media were broadly similar to those observed in the pond.

3.5. Metabolic adaptation to Cu tolerance by C. acidophila PM01

It was unknown whether the metal tolerance properties of *C. acidophila* differ between strains isolated from AMD sites with differing water chemistry. To begin to assess this, the tolerance of PM01 to a range of metals (Al, Cd, Cu, Fe, Mn and Zn) was compared to two other strains of *C. acidophila* (CCAP 11/136 and CCAP 11/137) that had previously been isolated from different field sites. Strains CCAP 11/136 and CCAP 11/137 were validated by 18S rDNA sequencing and phylogenetic analysis as *C. acidophila* species (Fig. 6). The CCAP 11/136 strain was originally isolated from a highly acidic (~pH 1.0) humic acid-rich peat water environment but the metal characteristics of the site were not described (Fott and McCarthy, 1964). In contrast, strain CCAP 11/137 originated from German mining Lake 111 at pH 2.6, very low total P (8 µg L^{-1}) and fairly high levels of Zn (0.75 mg L^{-1}) (Spijkerman et al., 2007a).

For Al, Cd and Mn there was no growth difference between the three strains. However, Fe treatment substantially inhibited growth of CCAP 11/136, while Zn treatment slightly inhibited growth of PM01 and CCAP 11/137 (Fig. S4). However, there was a very clear-cut difference in growth between the three strains following Cu exposure (Fig. 7). On solid media containing 130 mg L^{-1} Cu, the CCAP 11/137 strain was unable to grow, and the CCAP 11/136 strain grew very weakly in contrast to strong growth by the PM01 strain (Fig. 7A). In liquid media with addition of 0.0 mg L^{-1}, 6.5 mg L^{-1} and 3 mg L^{-1} growth rate and cell

density after 14 days was identical between all three strains. However, with 130 mg L^{-1} Cu addition, cell growth was unchanged for PM01 but both CCAP 11/136 and 11/137 strains were barely able to grow (Fig. 7B).

To examine whether there were any macromolecular changes within the strains in response to the increasing copper treatment, and to examine whether the different strains could be distinguished on the basis of their metabolic 'fingerprint', the FT-IR spectroscopy technique was used. FT-IR spectra were collect for replicates of each strain cultivated in the absence of added Cu or with the addition of 6.5 and 13 mg L^{-1} Cu (Fig. S5). In addition, PM01 was tested at the 130 mg L^{-1} concentration that inhibited growth of 11/136 and 11/137, thereby preventing FT-IR spectroscopy analysis of these strains at the higher Cu concentration. PCA of all FT-IR spectra showed that the CCAP 11/137 strain samples cluster separately from CCAP 11/136 and PM01 in all treatments, with the close clustering of spectra indicating that Cu addition did not significantly alter the metabolic fingerprint of CCAP 11/137 (Fig. 8A). Likewise Cu addition did not significantly alter the metabolic fingerprint of the CCAP 11/136 strain, with all samples clustering with the PM01 control samples. However, there was a clear difference in the FT-IR spectra-derived metabolic profile of PM01 samples following addition of increasing Cu concentrations, with the sample position within the PCA plot changing on the basis of both PC1 and PC2. At the highest (130 mg L^{-1}) Cu concentration all replicate PM01 samples are distinct from the control samples (Fig. 8A). The PC loading plots (Fig. 8B) indicate that along PC1 the spectral changes are based predominantly on increased abundance of amide peaks at 1655 and 1545 cm^{-1}, as well as a decrease in one of the carbohydrate peaks at approximately 1036 cm^{-1}.

4. Discussion

This study has demonstrated that while AMD substantially impacts biodiversity in aquatic environments, there is still substantial taxa abundance in these extreme locations. The scarcity of invertebrates across the ponds clearly indicates the severity of pollution at this

abandoned mine site. With the exception of the near-neutral pH Pond 3, invertebrate diversity was very poor in all ponds. Taxa diversity overall, including invertebrate diversity in particular, was strongly correlated with pH and with dissolved metal concentration, which was in line with expectations and previous studies (Courtney and Clements, 2002; Malmqvist and Hoffsten, 1999). Nevertheless, some invertebrate species can adapt to extreme AMD conditions (De Bisthoven et al., 2005; Deneke, 2000), and can be used as indicators of mine-waste pollution (Gray and Delaney, 2008). Here Chironomidae were observed in all ponds, including the very acidic Pond 1 and 4, and highly abundant in Pond 9, probably because this pond is fairly shallow and has fine sediment in contrast to the other ponds. Certain Chironomidae species are able to survive in highly acidic waters, such as the acid-tolerant *Chironomus acidophilus* that has been found nearby in the highly acidic (pH 2.4) and metal-rich Afon Goch river draining Parys Mountain (Michailova et al., 2009).

While Chironomidae were present in all ponds so there was also the consistent presence of chlorophyte microalgae, and in particular a strain confirmed as *C. acidophila*. The widespread occurrence of *C. acidophila* at Parys Mountain reflects the extremely acidic and metal-rich pond water and is consistent with other acidic and metal-rich sites (Fott and McCarthy, 1964; Gerloff-Elias et al., 2005; Hargreaves et al., 1975; Twiss, 1990). The widely differing *C. acidophila* abundance between ponds may reflect nutrient availability. AMD environments typically have very low productivity, due in part to low nutrients such as PO_4-P (Spijkerman et al., 2007a; Spijkerman et al., 2007b), and in this study nutrients, and in particular PO_4-P, were at low concentration in all but Pond 1. The low cell density of this species in Ponds 2 – 9, where PO_4-P concentrations were low suggests growth limitation due to low PO_4-P availability. In contrast, Pond 1 had very high PO_4-P and a high concentration of *C. acidophila* cells. Other studies have shown high PO_4-P levels associated with acidic lakes in areas with abundant PO_4-P-containing FeS minerals (Spijkerman, 2008); however, as Pond 2 is within 5 m of Pond 1 and has the same mineralogy but significantly lower PO_4-P levels, the high concentration of PO_4-P in Pond 1 is unlikely to be due to minerology of the surrounding area. It may be that the high PO_4-P concentration is due to a

523 eutrophication event, as there is evidence of construction waste deposition that is likely to
524 explain the PO_4-P entry into the pond.
525 The high microalgae productivity of Pond 1 is due almost exclusively to *C. acidophila*
526 abundance. Acidophilic microalgae present in the other ponds, such as *K. corcontica*, *E.*
527 *naegelii* and *E. mutabilis*, were absent from Pond 1. *E. naegelii* and other acid-tolerant
528 diatoms are increasingly used as indicator species for acid polluted environments (Zalack et
529 al., 2010) and have been previously found in surface sediment from one of the rivers
530 draining from the Parys Mountain site (Dean et al., 2013), as well as in acid pit lakes with
531 equivalent water chemistry (Geller, 2013). However, their apparent low abundance in these
532 ponds might partly be due to the sampling regime used here from the near-surface open
533 water rather than from the bottom of the ponds. *E. mutabilis* was observed in two of the nine
534 ponds (Ponds 5 and 6), which are both ~pH 3.0, a pH range that has been shown to be
535 preferential for this organism (Brake et al., 2001). Furthermore, other acidophilic microalgae
536 such as *Ochromonas* sp. and *Dunaliella* sp., that are widely abundant in many acidic sites
537 (Aguilera et al., 2006; Nixdorf et al., 1998), were not identified in Pond 1, or in any other
538 Parys Mountain ponds. That *C. acidophila* was the only microalgal species identified in Pond
539 1 is likely to be due to the extreme conditions that restricts microalgal diversity. One of these
540 factors appears to be water pH, and the *C. acidophila* strain studied here was particularly
541 acid tolerant. In waters with pH > 3.0, it has been previously observed that algal biodiversity
542 is generally higher (Smucker et al., 2014), yet the least acidic pond studied here, Pond 3 (pH
543 5.3), still had very low algal biodiversity and very low *C. acidophila* abundance, suggesting
544 that other factors are also important, such as nutrient availability or water chemistry.
545 Although the dissolved Fe, Zn, Cu and Al concentrations in Pond 1 did not exceed
546 the very high concentrations seen in some AMD lakes and rivers such as those of the
547 Iberian Pyrite Belt (Sánchez España et al., 2008), they did exceed those seen in mine pit
548 lakes in Germany, Poland, Australia and USA (Geller, 2013). The metal concentrations of
549 the shallow Pond 1 can thus be considered high. The strain of *C. acidophila* isolated from
550 Pond 1 is therefore not just extremely acid-tolerant, but can also tolerate high metal

concentrations. The PM01 strain showed substantial tolerance to Cd, Cu, and Zn. A comparison of PM01 to other genera of acid tolerant algae shows the Zn tolerance of PM01 was higher than that reported for *Chlorella protothecoides var. acidicola* isolated from AMD sites in a Spanish mine and *E. mutabilis* isolated from the adit flowing from Parys Mountain. However, both of these strains showed a higher Cu tolerance than PM01 (Ñancucheo and Johnson, 2012).

Other studies looking at metal tolerance in *C. acidophila* are fairly scarce, though a study looking at a putative strain of *C. acidophila* OU 030/a isolated from a volcanic acid lake also showed high tolerance to Cd, Cu and Zn in metal-rich minimal media (at pH 4.0) when compared to other algal species (Nishikawa and Tominaga, 2001). Furthermore, a strain of *C. acidophila* RT46 isolated from Río Tinto in Spain, showed unaffected photosynthetic activity in response to 0.5 mM Cu (~32 mg L^{-1} Cu) exposure (Olsson et al., 2015). A putative *C. acidophila* strain (UTCC 121) isolated from Cu contaminated soil was previously shown to tolerate up to 100 mg L^{-1} Cu. However, in contrast, a laboratory strain of *C. acidophila* (CCAP 11/96) was Cu sensitive, as was the non-acidophilic freshwater alga *Chlamydomonas reinhardtii* (Twiss, 1990), indicating that the *Chlamydomonas* genera is not intrinsically Cu tolerant. In this study we demonstrate that the *C. acidophila* strain isolated in this study (PM01) has higher Cu tolerance than strains (CCAP 11/136 and 11/137) from AMD field sites with less Cu pollution. This shows that the PM01 strain has adapted to the high dissolved Cu concentrations of Pond 1, rather than this species having innate Cu tolerance properties.

FT-IR spectroscopy analysis demonstrates that the tolerance of PM01 to copper is partly due to its ability to modulate its metabolism in response to increasing Cu exposure, as indicated by a dose-dependent change in spectra characteristics, which does not occur with the other *C. acidophila* strains. Examination of the FT-IR spectra indicates that this metabolic adaptation is predominantly due to protein increase and potential modification of protein secondary structure, as shown by significant increase in amide I peak height associated with C=O stretching, and in amide II peak height associated with N-H bending and C-N stretching

(Giordano et al., 2001). A previous study using FT-IR spectroscopy to examine sensitivity and subsequent acclimation of microalgae to wastewater treatment, also found that the acclimation process was coincident with a relative increase in amide I and amide II peak height (Osundeko et al., 2014). Moreover, this same study showed that particularly sensitive strains including *Chlamydomonas debaryana* and *Desmodesmus intermedius* exhibited accumulation of carbon storage products including glycerolipids and starch, while acclimated strains that could tolerate the wastewater conditions did not show this response. Likewise, other metabolic indicators of stress such as increased carbohydrate and lipid peaks within the FT-IR spectra were not observed in response to high Cu treatment in PM01.

Because Cu exerts toxicity in part through inhibition of key cellular processes including photosynthesis, either directly due to Cu binding or indirectly via accumulation of ROS (Jamers et al., 2013; Küpper et al., 2003; Sabatini et al., 2009), it would be expected that adaptive mechanisms would counteract these processes in some ways. Proteomic responses to Cu stress linked to Cu tolerance have previously been observed in other organisms. Cu exposure experiments in plants and fungi have observed increases in soluble protein that has been linked to induction of anti-oxidant enzymes (Cavalcanti Luna et al., 2015; Gao et al., 2008; Rout et al., 2013) while induction of Cu-binding proteins has been demonstrated in a Cu-tolerant variety of rice (Chen et al., 2015). Mechanisms of Cu tolerance in algae are not well understood, and may involve differential Cu uptake and internalisation in some cases (Levy et al., 2008). Although the molecular mechanisms of stress tolerance by *C. acidophila* are also poorly understood but there have been some recent insights. Heat shock proteins, which are a family of evolutionarily conserved stress tolerance molecular chaperones, have been previously found to increase in abundance in *C. acidophila* CCAP 11/137 in response to very low pH and metal-rich lake water treatment, partly in response to high Fe concentration (Gerloff-Elias et al., 2006; Spijkerman et al., 2007a). To date, no proteomic or enzymatic analysis has been performed in *C. acidophila* in response to Cu stress, but a transcriptomics approach observed differences in mRNA transcript profiles following Cu treatment in *C. acidophila* RT46. A range of gene transcripts

607 were up-regulated in response to Cu treatment including those involved in photosynthesis,
608 signaling and stress-response (Olsson et al., 2015). Future experiments will aim to examine
609 the proteomic adaptive response of *C. acidophila* PM01 in more detail in order to enhance
610 our fundamental understanding of metal tolerance in microalgae, also to appreciate the
611 effects that environmental pollution has on adaptive evolution and the ecological
612 consequences of such adaptation.

613 A potential application of highly metal tolerant microalgae such as *C. acidophila*
614 PM01 is the potential use of such organisms for metal bioremediation. Indeed PM01 was
615 confirmed to bioconcentrate metals through a combination of cell wall binding and
616 internalisation. Algae strains, such as those isolated in this study, may be used for *in situ*
617 lake bioremediation in surface water mesocosms by controlled eutrophication and harvesting
618 (Dessouki et al., 2005), or for *ex situ* bioremediation, such as immobilised algae in
619 bioreactors (Mehta and Gaur, 2005). The accumulated metals may then be harvested and
620 processed to allow metal recovery (Minoda et al., 2015; Raikova et al., 2016). Alternatively,
621 highly acid and metal tolerant microalgae such as the PM01 strain, may have an important
622 role in sustaining SO_4-reducing bacteria by providing organic carbon and thus increasing the
623 efficiency of AMD remediation microbial bioreactors (Diez-Ercilla et al., 2014; Ňancucheo
624 and Johnson, 2012; Totsche et al., 2006). For example, it was demonstrated that microalgal
625 addition to mine tailing mesocosms containing pyrite-oxidizing bacteria caused higher
626 production of alkalinity, higher concentrations of ferrous Fe, and increased immobilization of
627 Cu and Zn (Ňancucheo and Johnson, 2011).

628 The main aim of this study was to identify microorganisms from the open, standing
629 waters of the AMD ponds. Some of the ponds were surrounded by vegetation including
630 wetland plant species, which will harbor associated microorganisms (Aguinaga et al., 2018).
631 Although not the scope of this study, future research can examine the role of the plants on
632 microbial communities within isolated AMD pond environments, as well an examination of
633 biota along spatial transects of the ponds including within the sediment. Moreover, future
634 studies will be needed to examine how other microorganisms found in these environments

have adapted to AMD stresses, and whether there are common mechanisms between different extremophile species.

5. Conclusions

AMD is a major source of freshwater pollution worldwide for which restoration is very important. However, as found in this study, while extremely acidic and metal rich AMD substantially impacts biota, there is still substantial biodiversity, with tolerance derived through natural adaptation. In particular, a strain of *C. acidophila* is abundant in all ponds at this Cu mine site, especially in waters with high acidity and coupled with high PO_4-P concentration. Although Cu toxicity is a significant challenge to most photosynthetic organisms, this strain of *C. acidophila* has specifically adapted to the high Cu status of the ponds in contrast to other strains of the same species isolated from field sites elsewhere. Moreover, the *C. acidophila* strain displays evidence of Cu-dependent metabolic plasticity. The marked metal tolerance and metal accumulation characteristics of *C. acidophila* PM01 indicates that organisms from these environments have biotechnological potential, such as bioremediation.

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Figures

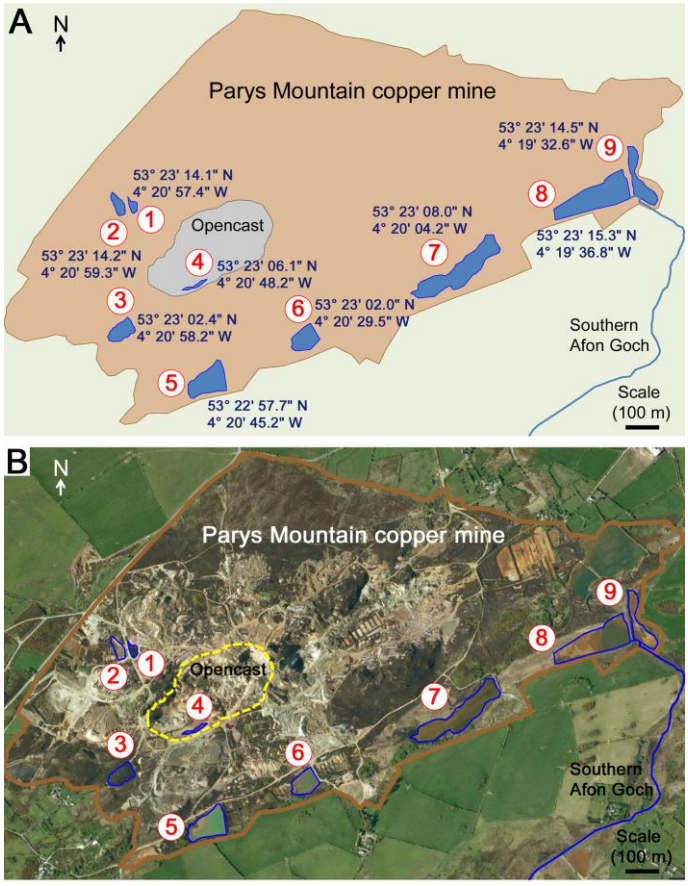
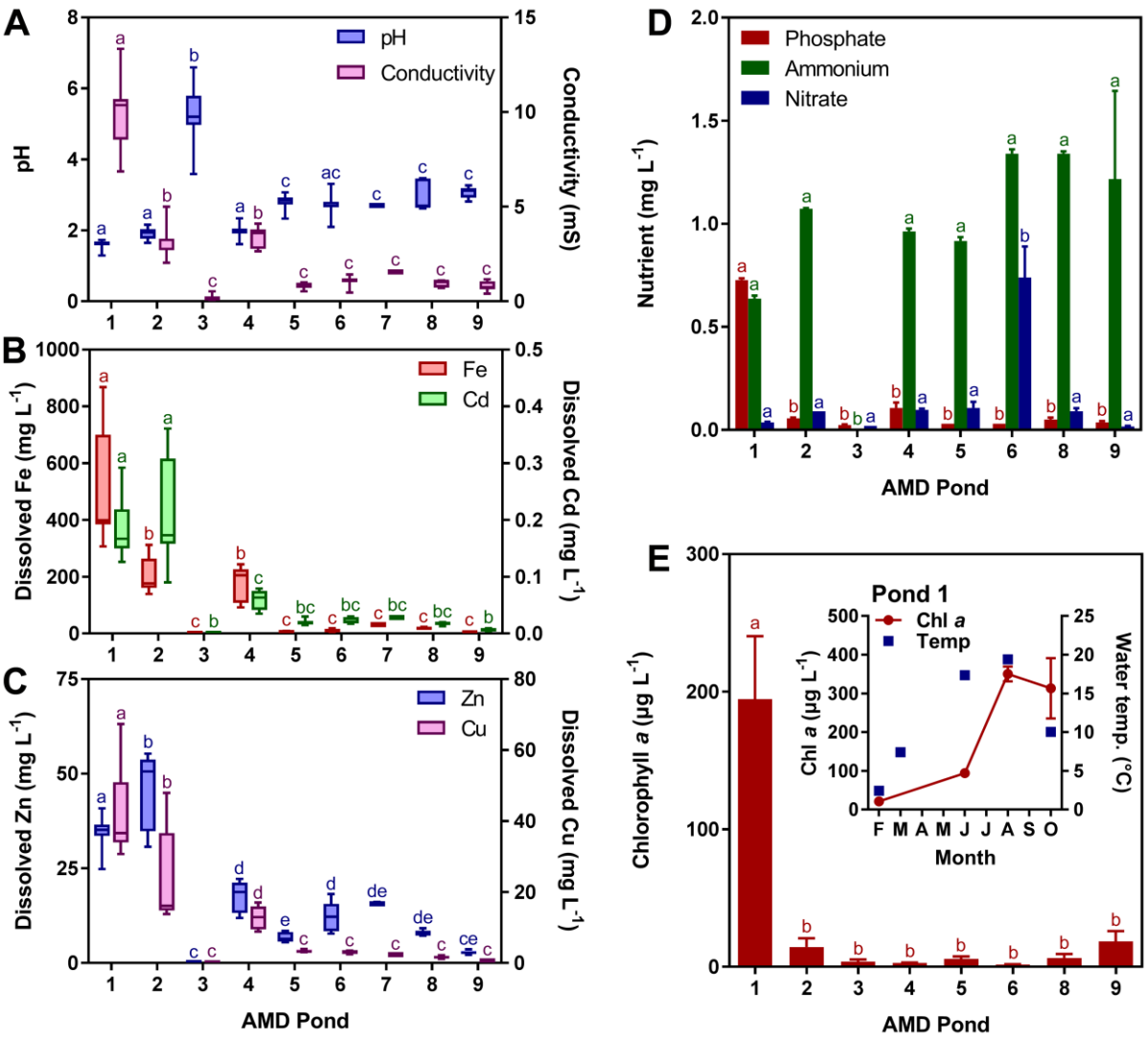


Fig. 1. Map and satellite image of Parys Mountain copper mine in Anglesey, North Wales, UK. The sampled AMD ponds are labelled 1−9 and the AMD-polluted Southern Afon Goch river is indicated. The location coordinates of each sampled pond are also shown (A). (B) Satellite image of the mine and the sampled ponds. Source, Google Earth, 2016.



928

929 Fig. 2. Water chemistry of the AMD ponds. (A - C) pH and conductivity values (A), and
930 concentration of dissolved Fe, Cd (B), Cu and Zn (C) for each pond during 2015 (spring,
931 summer, autumn samples). Boxes show the 25th and 75th percentiles, the line within the
932 boxes shows the median values, and the whisker bars show minimum and maximum values
933 (n = 3 – 18). (D) PO_4-P, NO_3-N and NH_4-N concentration in each pond (June sample). Pond
934 7 data is not available. Values are means (n = 3 - 12) and error bars correspond to the
935 standard error of the mean. (E) Mean chlorophyll-*a* (Chl *a*) concentration in each pond
936 during 2015 (spring, summer, autumn samples). Chl *a* change in Pond 1 during the year and
937 pond water temperature is shown (inset). Each pond was sampled in triplicate on three
938 separate occasions (spring, summer, autumn). Values are means (n = 9) and error bars
939 correspond to the standard error of the mean. For all data, bars that do not share a lower
940 case letter show significant difference ($p < 0.05$) between pond sites.
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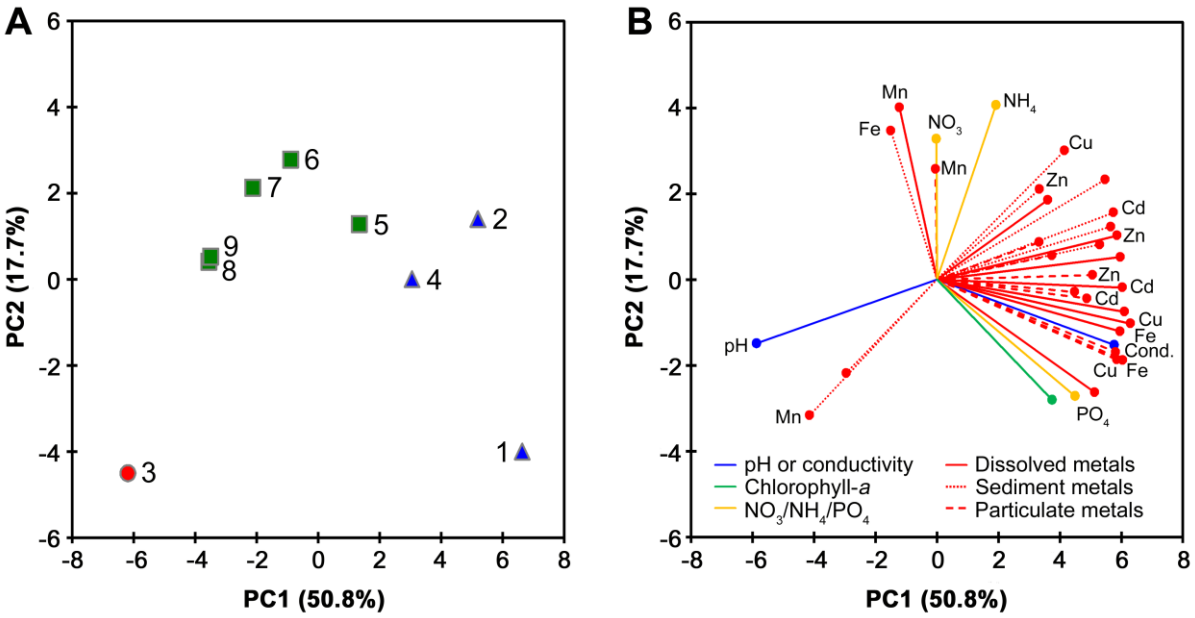


Fig. 3. PCA of water chemistry data from each of the AMD ponds. (A) Observation plot for each pond. The upper site small ponds are shown as blue triangles, the near-neutral pH pond as a red circle, and the lower site large lagoon ponds as green squares. (B) Variables plot with selected environmental variables labelled.

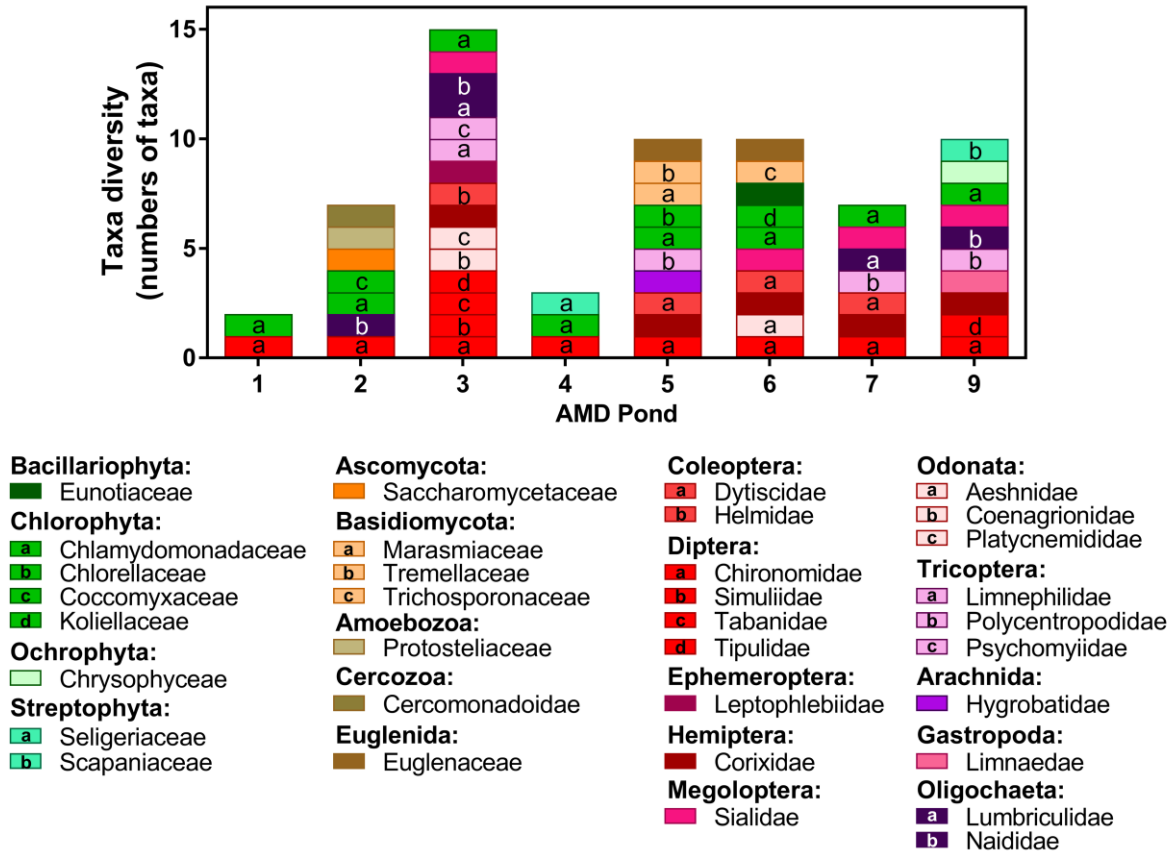
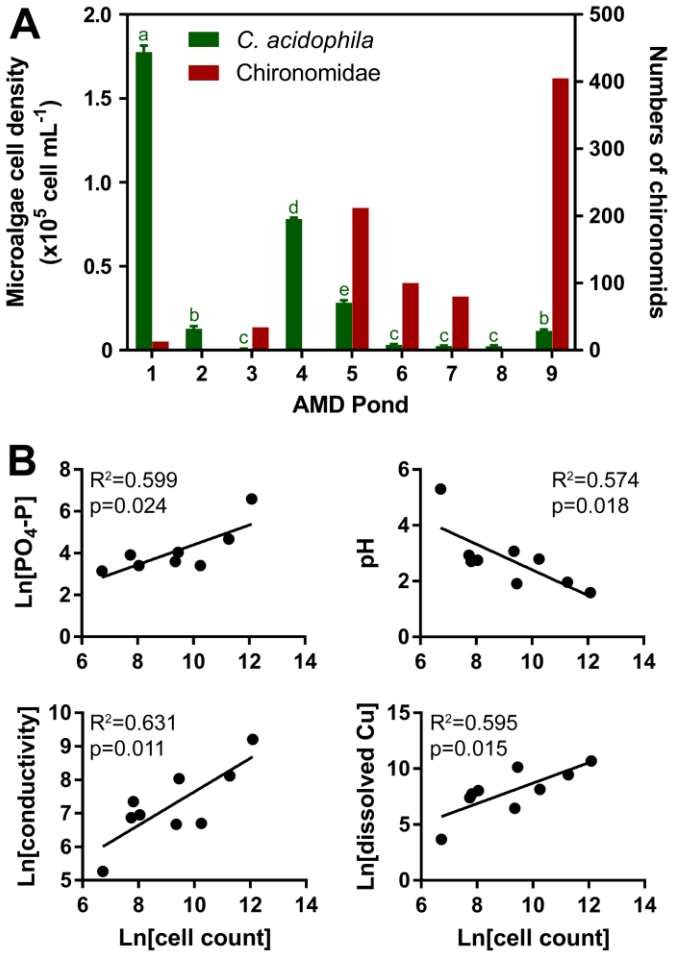


Fig. 4. Taxa diversity of eukaryotic organisms in the AMD ponds as determined by numbers of distinct taxonomic families of microalgae, moss/liverwort, fungi, protozoa, as identified by 18S rRNA gene amplicon sequencing, and families of invertebrates, as identified by visual observation and use of taxonomic identification keys. Data for Pond 8 is not available.



954

955 Fig. 5. (A) Abundance of *C. acidophila* microalgae and Chironomidae insects in each pond
956 from summer sampling. Values of *C. acidophila* are means (n = 3) and error bars correspond
957 to the standard error of the mean, while value of Chironomidae are total counts. For *C.*
958 *acidophila* data, bars that do not share a lower case letter show significant difference ($p <$
959 0.05) between pond sites. Chironomidae data for Pond 8 is not available. (B) Linear
960 regression analyses for dissolved phosphate, pH, conductivity and dissolved Cu in relation to
961 *C. acidophila* cell counts in AMD ponds. Apart from pH values, all data were natural log
962 transformed.
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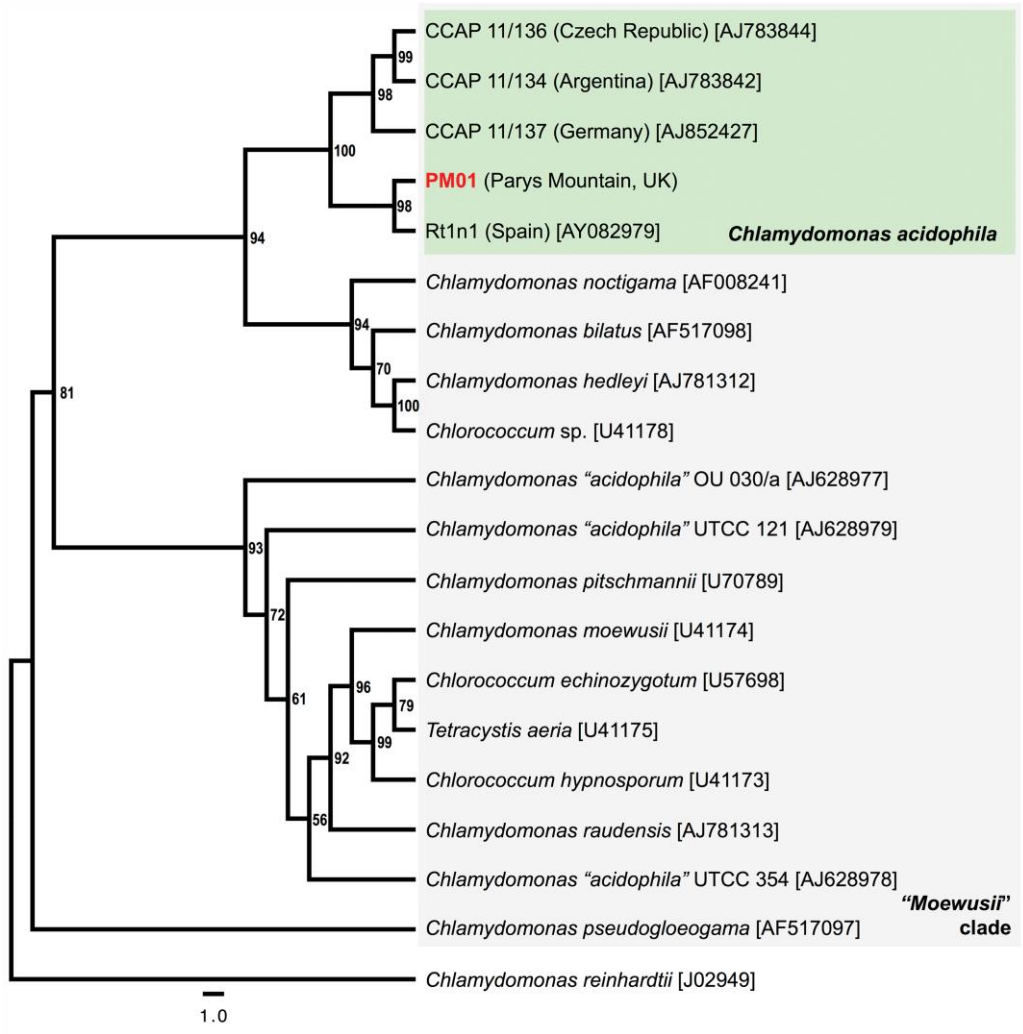


Fig. 6. 18S rDNA sequence analysis of *C. acidophila* strains. Phylogenetic tree based on 18S nucleotide sequence obtained from the PM01 strain isolated from Pond 1, sequences of *C. acidophila* CCAP 11/136 and CCAP 11/137 strains, other known or putative *C. acidophila* strains, and selected unicellular Chlorophyta microalgae of the *Chlamydomonas moewusii* clade, including three strains originally classified as *C. acidophila*. The sequence lengths were between 1528 – 1792 nucleotides. *Chlamydomonas reinhardtii* is included as an out-group. Accession numbers are shown for each sequence. Bootstrap percentage values are indicated at the tree nodes of branches for 100 replications and indicate confidence in tree node positions. The branch length scale bar indicates evolutionary distance.

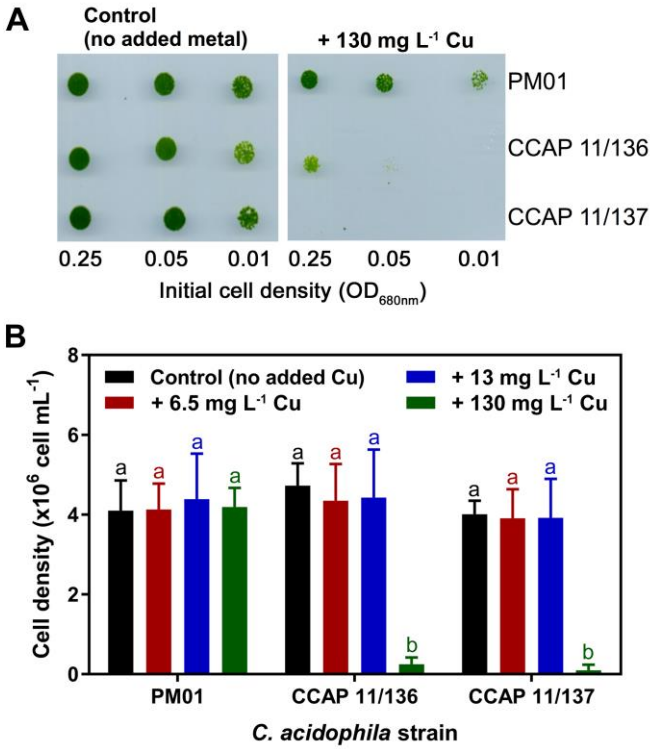
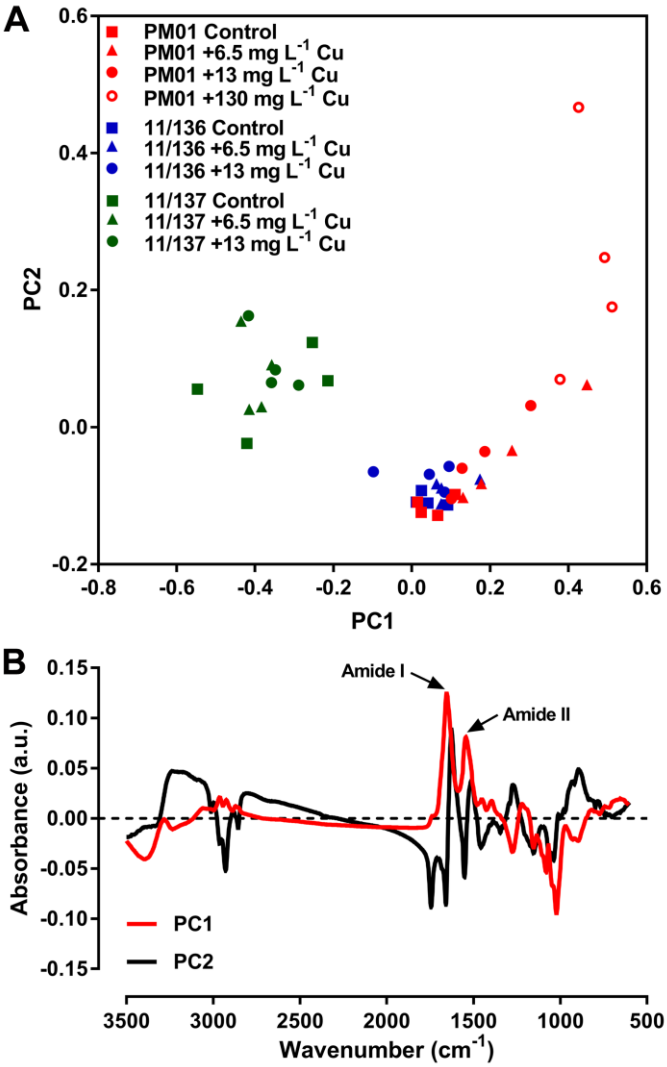


Fig. 7. Cu tolerance of *C. acidophila* PM01 in comparison to *C. acidophila* strains CCAP 11/136 and 11/137. (A) Growth of algae spot dilutions on MAM pH 3.0 plates with or without added Cu and photographed after 12 d. Image is representative of 3 independent experiments. (B) Growth of strains in liquid MAM pH 3.0 determined after 14 d cultivation in response to a range of Cu concentrations. Values are means (n = 3 - 4) and error bars correspond to the standard error of the mean. Bars that do not share a lower case letter show significant difference ($p < 0.05$) between strains.



984

985 Fig. 8. PCA clustering of FT-IR spectra from *C. acidophila* PM01 in comparison to *C.*
986 *acidophila* strains CCAP 11/136 and 11/137 in response to a range of Cu concentrations. (A)
987 PCA scores plot of replicate (n = 4) FT-IR spectra obtained from cells grown after 14 d
988 cultivation in liquid MAM pH 3.0 with increasing concentrations of added Cu or no added Cu.
989 Only PM01 cells could grow in 130 mg L^{-1} Cu. (B) PC1 and PC2 loading plot. Band peaks
990 which explain most of the variation for each PC are υC=O of amide I (1655 cm^{-1}) and δN-H
991 of amide II (1545 cm^{-1}).
992